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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,230,203,** on April 29, 1998, by **UNIVERSITE DE SHERBROOKE**, assignee of Jean-Pierre Perreault, Sirinart Ananvoranich and Daniel Lafontaine, for "Delta Ribozyme for RNA Cleavage".

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ABSTRACT OF THE INVENTION

The present invention relates to an enzymatic ribonucleic acid molecule consisting in a ribonucleotide sequence having an RNA substrate-cleaving enzymatic activity to cleave a separate RNA substrate at a cleavage site, which comprises: a) an RNA substrate binding portion base pairing with the RNA substrate only 3' of the cleavage site in two consecutive RNA duplex having three Watson-Crick base pairs, wherein the two consecutive RNA duplex are separated by one pyrimidine forming a non-conventional Watson-Crick base pair with a purine of the ribozyme, and wherein said pyrimidine or said purine is involved in a tertiary interaction with a third nucleotide of the ribozyme; b) an RNA substrate with a first nucleotide located 5' of the cleavage site is selected for the group consisting of U, A and G, and the first four nucleotides located 5' of the cleavage site remain single-stranded; c) an enzymatic portion including part or all of the RNA substrate binding portion and having the enzymatic activity located 3' and/or 5' of the RNA substrate binding portion; whereby base pairing of the enzymatic ribonucleic acid molecule with the separate RNA substrate causes cleavage of the RNA substrate at the cleavage site.

DELTA RIBOZYME FOR RNA CLEAVAGE

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a novel *delta* ribozyme construction for the specific recognition and cleavage of RNA, and therapeutic uses thereof.

(b) Description of Prior Art

Discoveries in the basic realm of molecular biology over the past ten years have led to the realization that RNA has a series of distinct capabilities and biological activities previously unsuspected. The most important of these novel RNA-level discoveries has been the finding that RNA can be an enzyme as well as an information carrier.

Various RNA molecules have one or more enzymatic activities, such as an endoribonuclease activity which acts to cleave other RNA molecules. Such activity is termed intermolecular cleaving activity. These enzymatic RNA molecules are derived from an RNA molecule which has an activity which results in its own cleavage and splicing. Such self-cleavage is an example of an intramolecular cleaving activity.

Since 1982, several unexpected diseases caused by RNA-based pathogenic agents have emerged. These include the lethal Acquired Immune Deficiency Syndrome (AIDS) and delta hepatitis, a particularly virulent form of fulminant hepatitis caused by a viroid-like RNA agent. These blood-borne diseases are spread at the RNA level, manifest themselves in cells of patients, and are by now present within the bloodstream of millions of individuals.

Conventional biotechnology, with its reliance on recombinant DNA methods and DNA-level intervention schemes, has been slow to provide valid approaches to combat these diseases.

Delta ribozymes, derived from the genome of hepatitis *delta* virus (HDV), are metalloenzymes. Like other catalytically active ribozymes, namely hammerhead and hairpin ribozymes, the delta ribozymes cleave a phosphodiester bond of their RNA substrates and give rise to reaction products containing a 5'-hydroxyl and a 2',3'-cyclic phosphate termini. Two forms of delta ribozymes, namely genomic and antigenomic, were derived and referred to by the polarity of HDV genome from which the ribozyme was generated. Both HDV strands forms exhibit self-cleavage activity, and it has been suggested that they are involved in the process of viral replication (Lazinski, D. W., and Taylor, J. M. (1995) *RNA* **1**, 225-233). This type of activity is described as cis-acting delta ribozymes.

Like other ribozymes, delta ribozymes have a potential application in gene therapy in which an engineered ribozyme is directed to inhibit gene expression by targeting either a specific mRNA or viral RNA molecule. A very low concentration (< 0.1 mM) of Ca^{2+} and Mg^{2+} is required for *delta* ribozyme cleavage.

In United States Patent No. 5,225,337, issued on July 6, 1993 in the names of Hugh D. Robertson et al., there is disclosed ribozymes derived from a specific domain present in the hepatitis delta virus (HDV) RNA for specifically cleaving targeted RNA sequences and uses thereof for the treatment of disease conditions which involve RNA expression, such as AIDS. These ribozymes consist in at least 18 consecutive nucleotides from the conserved region of the hepatitis delta virus between residues 611 and 771 on the genomic strand and between residues 845 and 980 on the complementary anti-genomic strand. These ribozymes are proposed to fold into an axe-head model secondary structure (Branch, A. D., and Robertson, H. D. (1991)

Proc. Natl. Acad. Sci. USA **88**, 10163-10167). According to this model structure, these ribozymes require substrate base paired by 12-15 nucleotides, which size is not clinically practical, in order to exhibit the desired activity. More specifically, a substrate bound to the ribozyme through the formation of two helices. A helix is located upstream to the cleavage site (e.g. in 5' position) while the second helix is located downstream to the cleavage site (e.g. in 3' position).

In United States Patent No. 5,625,047, issued on April 29, 1997 in the names of Michael D. Been et al., there is disclosed enzymatic RNA molecules proposed to fold into a pseudoknot model secondary structure. These ribozymes were proposed to cleave at almost any 7 or 8 nucleotide site having only a preference for a guanosine base immediately 3' to the cleavage site, a preference for U, C or A immediately 5' to the cleavage site, and the availability of a 2' hydroxyl group for cleavage to occur. The specificity of recognition of these ribozymes is limited to 6 or 7 base pairing nucleotides with the substrate and a preference of the first nucleotide located 5' to the cleavage site. Neither tertiary interaction(s) between the base paired nucleotides and another region of the ribozyme, nor single-stranded nucleotide are involved to define the specificity of recognition of these ribozymes. Because the recognition features were included in a very small domain (e.g. 6 or 7 base paired nucleotides) in order to exhibit the desired activity, these ribozymes have a limited specificity, and thus, not practical for further clinical applications.

It would be highly desirable to be provided with a novel *delta* ribozyme for the cleavage of both small and large RNA substrates for which the

specificity is defined by a domain composed of at least 10 nucleotides. Such specificity would yield to optimal conditions for further therapeutical developments of delta ribozymes.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide with a novel *delta* ribozyme for the cleavage of RNA substrates for which the specificity is defined by a domain composed of at least 10 nucleotides. The substrate recognition mechanism of this ribozyme is unique involving formation of base pairs, requirement for single-stranded nucleotide as well as at least one crucial tertiary interaction.

In accordance with the present invention there is provided an enzymatic ribonucleic acid molecule consisting in a ribonucleotide sequence having an RNA substrate-cleaving enzymatic activity to cleave a separate RNA substrate at a cleavage site, which comprises:

a) an RNA substrate binding portion base pairing with the RNA substrate only 3' of the cleavage site in two consecutive RNA duplex having three Watson-Crick base pairs, wherein the two consecutive RNA duplex are separated by one pyrimidine forming a non-conventional Watson-Crick base pair with a purine of the ribozyme, and wherein said pyrimidine or said purine is involved in a tertiary interaction with a third nucleotide of the ribozyme;

b) an RNA substrate with a first nucleotide located 5' of the cleavage site is selected for the group consisting of U, A and C, and the first four nucleotides located 5' of the cleavage site remain single-stranded ;

c) an enzymatic portion including part or all of the RNA substrate binding portion and having the enzymatic activity located 3' and/or 5' of the RNA substrate binding portion;

whereby base pairing of the enzymatic ribonucleic acid molecule with the separate RNA substrate causes cleavage of the RNA substrate at the cleavage site.

The ribonucleotide sequence may be derived from hepatitis delta virus.

The RNA substrate include, without limitation, a sequence H↓GNNY'NN (for a 6 base pairs binding domain) or H↓GNNY'NNN (for a 7 base pairs binding domain),

wherein,

N is independently a nucleotide base forming Watson-Crick base pairs with the RNA substrate binding portion,

↓ is the cleavage site;

H is one base selected from the group consisting of A, U and C, wherein H is adjacent to the cleavage site and not base pairing to the ribozyme; and

Y' is a pyrimidine base (U or C) forming a non-conventional Watson-Crick base pair with the RNA ribozyme binding portion.

The preferred RNA substrate sequence do not carry two consecutive pyrimidine (U or C) in the first four nucleotides 5' to the cleavage site.

The preferred RNA substrate sequence may be selected from the group consisting of GGGC↓GNNUNNN, GGGC↓GNNCNNN, GGGU↓GNNUNNN, GGGU↓GNNCNNN, AAAC↓GNNUNNN (for 11 bases long substrate, as example only).

The RNA ribozyme binding portion which base pairs with the RNA substrate 3' has the sequence

NNR'NNU (for 10 base substrate) or , NNNR'NNU (for 11 base substrate).

wherein,

N is independently a nucleotide base forming Watson-Crick base pairs with the substrate, and

R' is a purine base forming a non-conventional Watson-Crick base pair with the substrate. The preferred RNA ribozyme binding portion may be selected from the group consisting of (5' to 3') NNNANNU, NNNGNNU (for 11 base long substrate, as example only).

For the purpose of the present invention the following abbreviations are defined below.

"A" is an adenine base

"G" is a guanine base

"C" is a cytidine base

"U" is a uracil base

"R" is a purine, which is a base selected from the group consisting of A or G.

"Y" is a pyrimidine, which is a base selected from the group consisting of U and C.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates secondary structure and nucleotide sequences of the constructed *trans*-acting antigenomic *delta* ribozymes and their complementary substrates;

Fig. 2 illustrates time course for a cleavage reaction catalyzed by δRzP1.1;

Fig. 3 illustrates kinetics of cleavage reactions catalyzed by *trans*-acting *delta* ribozymes;

Fig. 4 illustrates the measurement of equilibrium dissociation constant (K_d);

Fig. 5 illustrates free energy diagrams for cleavage reactions catalyzed by δRzP1.1 and δRzP1.2;

Figs. 6A-B illustrate the studied delta ribozyme (Fig. 6A) and the substrate specificity (Fig. 6B); and

Figs. 7A-C illustrate delta ribozymes cleaving various non-natural substrates.

DETAILED DESCRIPTION OF THE INVENTION

Although one might suspect the specificity of delta ribozyme cleavages due to their short recognition site, we view this characteristic of the *delta* ribozyme as an advantage for the future development of a therapeutic means of controlling, for example, a viral infection.

One distinctive characteristic of the delta ribozyme is that it remains catalytically active even in the presence of low concentrations of either calcium or magnesium (e.g. < 1 mM).

Since little is known about the kinetic properties of *delta* ribozymes, study of the trans-acting system will enable us to answer some basic questions on both the structure required and the kinetic properties, including the substrate specificity, of delta ribozymes.

In accordance with the present invention, a trans-acting delta ribozyme was generated based on the pseudoknot-like structure proposed by Perrotta and Been (Perrotta, A.T., and Been, M. D. (1991) *Nature* **350**, 434-436), by separating the single-stranded region located at the junction between the P1 and P2 stems (Fig. 1). The base paired regions of the pseudoknot-like structure are numbered according to Perrotta and Been (Perrotta, A.T., and Been, M. D. (1991) *Nature* **350**, 434-436). *Panel* **A**, secondary structure of the complex formed between δRzP1.1 and its substrate, SP1.1. The arrow indicates the cleavage site. The nucleotide numbering of the substrate is indicated and

referred to throughout the text. The dotted line represents a single-strand region joining the substrate and ribozyme molecules as presented in the *cis*-form (namely J1/2). This single-strand area was eliminated to produce the *trans*-acting ribozymes used in this study. *Panel* ***B***, The P1 region of δRzP1.2 and its substrate, SP1.2. Two base pairs at the positions 7 and 9 of the substrate and positions 22 and 24 of δRzP1.1 were swapped. δRzP1.2 was constructed using of the *Rsr*II and *Sph*I restriction sites indicated on the structure of δRzP1.1 as described in Materials and Methods. The rest of the structure is identical to δRzP1.1 as in panel ***A***.

Although, several investigations have been performed to address the questions related to the substrate specificity of delta ribozymes in both the cis- and trans-acting forms (Been, M. D. (1994) *TIBS* **19**, 251-256), most, if not all, experiments were carried out by randomly changing the base pairing combinations, or by introducing mismatches which interfere with the Watson-Crick base pairing between the substrate and the ribozyme in the P1 stem (Fig. 1). It was demonstrated that cleavage activity was not destroyed by the interchanging of one to four nucleotide pairs between the substrate and the delta ribozyme. One or two nucleotide mismatches at any position of the P1 stem, except positions 5 and 11 (numbering according to Fig. 1), completely destroyed the activity. Although these are composite results from various versions of delta ribozymes, these findings could be interpreted as indicating that the positions located at both extremities of the base paired stem formed by the substrate and the ribozyme were more likely to tolerate a mismatch, resulting distortion of the P1 stem, than the internal positions.

To date, there is no information on how each nucleotide of the substrate affects the cleavage activity and its kinetics since most investigations were carried out at only one or two positions at a time, and the findings generally reported in a plus/minus manner (e.g. cut or uncut). Therefore, the substrate specificity of delta ribozyme could not be deduced from previous reports.

In order to determine how substrate sequences affect delta ribozyme cleavage activity, we performed kinetic studies using a collection of short oligonucleotide substrates (11 nt) with a trans-acting delta ribozyme. In accordance with the present invention, it is demonstrated that each nucleotide of the P1 stem contributes differently to the cleavage activity. Observed cleavage rate constants for cleavable substrates, and the equilibrium dissociation constants for the uncleavable substrates, were compared to those of the wild type substrate. Evidence is presented that strongly suggests that the nucleotides located in the center of the P1 stem formed between substrate and ribozyme (Fig. 1, positions 7 and 8) are important not only for substrate recognition, but probably also for subsequent steps, for example a conformation change yielding a transition complex.

Plasmids carrying *delta* ribozymes

The antigenomic ribozyme sequence of the hepatitis *delta* virus described by Makino *et al* (Makino, S. et al. (1987) *Nature* **329**, 343-346) was used to generate a *trans*-acting *delta* ribozyme with some modifications as shown in Fig. 1. Briefly, the construction was performed as follows. Two pairs of complementary and overlapping oligonucleotides, representing the entire length of the ribozyme (57 nt), were synthesized and subjected to an annealing process

prior to cloning into pUC19. The annealed oligonucleotides were ligated to *Hind* III and *Sma* I co-digested pUC19 to give rise to a plasmid harboring the *delta* ribozyme (referred to as pδRzP1.1). A mutant ribozyme (δRzP1.2) was then constructed by modifying the substrate recognition site of pδRzP1.1 by ligation of an oligonucleotide containing the altered sequence flanked by restriction endonuclease sites to *Rsr* II/*SpH* I predigested pδRzP1.1. The sequences of engineered ribozymes were confirmed by DNA sequencing. Plasmids containing wild type and mutant ribozymes were then prepared using Qiagen tip-100 (Qiagen Inc.), digested with *Sma* I, purified by phenol and chloroform extraction and precipitated for further use as templates for *in vitro* transcription reactions.

RNA synthesis

Ribozyme: *In vitro* transcription reactions contained 5 µg linearized recombinant plasmid DNA as template, 27 units RNAGuard® RNase inhibitor (Pharmacia), 4 mM of each rNTP (Pharmacia), 80 mM HEPES-KOH pH 7.5, 24 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT, 0.01 unit Pyrophosphatase (Boehringer Mannheim) and 25 µg purified T7 RNA polymerase in a final volume of 50 µL, and were incubated at 37°C for 4 hr.

Substrates: Deoxyoligonucleotides (500 pmoles) containing the substrate and the T7 promoter sequence were denatured by heating at 95°C for 5 min in a 20 µL mixture containing 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM KCl_2, and allowed to cool slowly to 37°C. The *in vitro* transcription reactions were carried out using the resulting partial duplex formed as template under the same conditions as described for the production of the ribozyme.

After incubation, the reaction mixtures were fractionated by denaturing 20% polyacrylamide gel

electrophoresis (PAGE, 19:1 ratio of acrylamide to bisacrylamide) containing 45 mM Tris-borate pH 7.5, 7 M urea and 1 mM EDTA. The reaction products were visualized by UV shadowing. The bands corresponding to the correct sizes of either ribozymes or substrates were cut out, and the transcripts eluted overnight at 4°C in a solution containing 0.1% SDS and 0.5 M ammonium acetate. The transcripts were then precipitated by the addition of 0.1 vol 3 M sodium acetate pH 5.2 and 2.2 vol ethanol. Transcript yield was determined by spectrophotometry.

End-labeling of RNA with [γ-^{32}P]ATP

Purified transcripts (10 pmoles) were dephosphorylated in a 20 µL reaction mixture containing 200 mM Tris-HCl pH 8.0, 10 units RNA guard®, and 0.2 unit calf intestine alkaline phosphatase (Pharmacia). The mixture was incubated at 37°C for 30 min, and then extracted twice with a same volume of phenol:chloroform (1:1). Dephosphorylated transcripts (1 pmole) were end-labeled in a mixture containing 1.6 pmole [γ-^{32}P]ATP, 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM KCl and 3 units T4 polynucleotide kinase (Pharmacia) at 37°C for 30 min. Excess [γ-^{32}P]ATP was removed by applying the reaction mixture onto a spin column packed with a G-50 Sephadex™ gel matrix (Pharmacia). The concentration of labeled transcripts was adjusted to 0.01 pmol per ml by the addition of water.

Cleavage reactions

To initiate a cleavage reaction, we tested different procedure and chose the method which yielded the highest cleavage rate constant and the maximum cleavage product. Various concentrations of ribozymes were mixed with trace amounts of substrate (final concentration < 1 nM) in a 18 µL reaction mixture containing 50 mM Tris-HCl pH 7.5, and subjected to

denaturation by heating at 95°C for 2 min. The mixtures were quickly placed on ice for 2 min and equilibrated to 37°C for 5 min prior to the initiation of the reaction. Unless stated otherwise, cleavage was initiated by the addition of $MgCl_2$ to 10 mM final concentration. The cleavage reactions were incubated at 37°C, and followed for 3.5 hours or until the endpoint of cleavage was reached. The reaction mixtures were periodically sampled (2-3 µL), and these samples were quenched by the addition of 5 µL stop solution containing 95% formamide, 10 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. The resulting samples were analyzed by a 20% PAGE as described above. Both the substrate (11 nt) and the reaction product (4 nt) bands were detected using a Molecular Dynamic radioanalytic scanner after exposition of the gels to a phosphoimaging screen.

Kinetic analysis

Measurement of pseudo-first-order rate constant (k_{cat}, K_M and k_{cat}/K_M)

Kinetic analyses were performed under single turnover conditions. Trace amounts of end-labeled substrate (<1 nM) were cleaved by various ribozyme concentrations (5 to 500 nM). The fraction cleaved was determined, and the rate of cleavage (k_{obs}) obtained from fitting the data to the equation $A_t = A_\infty(1-e^{-kt})$ where A_t is the percentage of cleavage at time t, A_∞ is the maximum percent cleavage (or the end point of cleavage), and k is the rate constant (k_{obs}). Each rate constant was calculated from at least two measurements. The values of k_{obs} obtained were then plotted as a function of ribozyme concentrations for determination of the other kinetic parameters: k_{cat}, K_M and k_{cat}/K_M. Values obtained from independent experiments varied less than 15%. The requirement for Mg^{2+} by both

ribozymes was studied by incubating the reaction mixtures with various concentrations of $MgCl_2$ (1 to 500 mM) in the presence of an excess of ribozyme (500 nM) over substrate (< 1nM). The concentrations of Mg^{2+} at the half maximal velocity were determined for both ribozymes.

Determination of equilibrium dissociation constants (K_d)

For mismatched substrates which could not be cleaved by the ribozyme, the equilibrium dissociation constants were determined as follows. Eleven different ribozyme concentrations, ranging from 5 to 600 nM, were individually mixed with trace amounts of end-labeled substrates (< 1nM) in a 9 µL solution containing 50 mM Tris-HCl pH 7.5, heated at 95°C for 2 min and cooled to 37°C for 5 min prior to the addition of $MgCl_2$ to a final concentration of 10 mM, in a manner similar to that of a regular cleavage reaction. The samples were incubated at 37°C for 1.5 h, at which 2 µL of sample loading solution (50% glycerol, 0.025% of each bromophenol blue and xylene cyanol) was added, and the resulting mixtures were electrophoresed through a nondenaturing polyacrylamide gel (20% acrylamide with a 19:1 ratio of acrylamide to bisacrylamide, 45 mM Tris-borate buffer pH 7.5 and 10 mM $MgCl_2$). Polyacrylamide gels were pre-run at 20 W for 1 h prior to sample loading, and the migration was carried out at 15 W for 4.5 h at room temperature. Quantification of bound and free substrates was performed following an exposure of the gels to a phosphoimaging screen as described earlier.

Results

The *trans*-acting *delta* ribozymes illustrated herein were derived from the antigenomic *delta* ribozyme of HDV (Makino, S. et al. (1987) *Nature* **329**, 343-346).

Some features of the antigenomic *delta* ribozyme were modified to improve its structural stability and to aid in transcript production. Based on a pseudoknot-like structure described by Perrotta and Been (Perrotta, A.T., and Been, M. D. (1991) *Nature* **350**, 434-436), Fig. 1 shows the structure of the *delta* ribozymes used, with some modifications: i) the single-stranded region between substrate and ribozyme (region J1/2) was eliminated in order to separate the substrate molecule from the ribozyme; ii) the substrate contains only 11 nt and produces 7-nt and 4-nt cleavage products, and the GGG at the 5'-end was added in order to increase the yield during *in vitro* transcription; iii) three G-C base pairs were introduced in the P2 region to improve both the structural stability and transcript production; and iv) the P4 stem was shortened. Prior to performing a cleavage reaction, native gel electrophoresis was used to test for the possible presence of aggregates or multimer forms of the transcripts. Various concentrations of ribozyme, ranging from 5 nM to 2 μM, were mixed with trace amounts of end-labeled ribozyme (less than 0.5 nM) and fractionated under nondenaturing conditions as described in Materials and Methods. We detected the presence of a slow migrating species of ribozyme in the mixture containing 2 μM ribozyme. The quantification of the slow migrating band showed that the band amounted to approximately 2% of the total radioactive material. However, a single band was detected at the concentrations used for kinetic analysis and under single turnover conditions (5 to 600 nM). Similar experiments were performed for each substrate. There was no substrate multimer detected at the concentrations used. The equimolar mixture of end-labeled substrate and ribozyme was also fractionated

under nondenaturing conditions, and it resulted a single band of ribozyme and substrate complex similar to those observed for the K_d measurement shown in Fig. 4.

Cleavage kinetics of constructed antigenomic *delta* ribozymes

Two forms of *trans*-acting *delta* ribozymes (δRzP1.1 and δRzP1.2) were used with their corresponding substrates (11 nt) for the kinetic studies. δRzP1.2 differs from δRzP1.1 in that δRzP1.2 has two nucleotides, at positions 22 and 24 of δRzP1.1, interchanged (5'-CCCAGCU-3', Fig. 1). Time course experiments for cleavage reactions catalyzed by both δRzP1.1 and δRzP1.2 were monitored by the appearance of the 4 nt cleavage product. An example of a time course experiment for a cleavage reaction catalyzed by δRzP1.1 is shown in Fig. 2, panel A. *Panel* **A.** An autoradiogram of a polyacrylamide gel showing a cleavage assay carried out under single turnover conditions as described in Materials and Methods. One hundred nM δRzP1.1 were incubated with 1 nM end-labeled SP1.1 at 37°C in the presence of 50 mM Tris-HCl pH 7.5 and 10 mM $MgCl_2$. The positions of xylene cyanol (XC) and bromophenol blue (BPB) are indicated. *Panel* **B.** Quantification of the data in (A). A single exponential equation was used to fit data to k_{obs} = 0.21 min^{-1} and 68% as the extent of cleavage.

In this particular experiment, 100 nM of δRzP1.1 was incubated with 1 nM end-labeled substrate, SP1.1. The newly formed product and the remaining substrate bands at each time point were quantified, and the percentage of cleavage was plotted as a function of time (Fig. 2, panel B). δRzP1.1 cleaved approximately 60% of the substrate within 10 min. The data were fitted to a single exponential equation as described in

Materials and Methods so as to obtain the observed rate constant (k_{obs} = 0.21 min^{-1}). We attempted to fit the data as biphasic reactions. We observed that the standard deviation (χ^2) of data fitted to a double-exponential equation was higher (χ^2 = 0.01203) than that fitted to a single exponential equation (χ^2 = 0.000203). Although we could not exclude or dismissed completely the possibility that more than one conformation of the active ribozyme could be formed, the data were treated as if the reactions were monophasic in their kinetics for comparison purposes.

Similar experiments were performed using trace amounts of substrate (<1 nM) and various ribozyme concentrations in order to measure k_{obs} at each ribozyme concentration. The values of k_{obs} of both δRzP1.1 and δRzP1.2 increased with an increase in ribozyme concentration up to approximately 200 nM (Fig. 3, panel A). *Panel* **A**. The observed cleavage rate constants were plotted as a function of ribozyme concentrations. The values of δRzP1.1 indicate by ●; those of δRzP1.2 by ○. The concentrations of ribozyme at half velocity are 17.9 ± 5.6 nM for δRzP1.1 and 16.7 ± 6.4 nM for δRzP1.2. The values of k_{cat} is 0.34 min^{-1} for δRzP1.1 and 0.13 min^{-1} for δRzP1.2. Standard error from two independent experiments were less than 15%. *Panel* **B**. The requirement of Mg^{2+} for cleavage reactions catalyzed by δRzP1.1 and δRzP1.2. Under single turnover conditions, 500 nM ribozymes were incubated with 1 nM end-labeled complementary substrates in presence of various concentration of Mg^{2+}. The obtained initial cleavage rates were plotted as a function of the Mg^{2+} concentrations. The values of δRzP1.1 indicate by ●; those of δRzP1.2 by ○. At 10 mM Mg^{2+}, which was used in a regular cleavage reaction, both ribozymes cleaved

their substrates at their maximum rate (0.3 min^{-1} for δRzP1.1 and 0.1 min^{-1} for δRzP1.2).

The concentration of ribozyme at which the reaction velocity reached half-maximal (apparent K_M, K_M') is 17.9 ± 5.6 nM for δRzP1.1 and 16.7 ± 6.4 nM for δRzP1.2. Under the reaction conditions used, in which the increase in ribozyme concentration has no significant effect on the rate of cleavage, the cleavage rate (k_{obs}) is therefore represented by the catalytic rate constant (k_{cat}). The cleavage rate constants are 0.34 min^{-1} for δRzP1.1 and 0.13 min^{-1} for δRzP1.2. Apparent second-order rate constants (k_{cat}/K_M') were calculated to be 1.89 x 10^7 $min^{-1}·M^{-1}$ for δRzP1.1 and 0.81 x 10^7 $min^{-1}·M^{-1}$ for δRzP1.2 (Table 1).

Table 1

Kinetic parameters of wild type ribozyme (δRzP1.1) and mutant ribozyme (δRzP1.2)

Kinetic parameters	δRzP1.1	δRzP1.2
k_{cat} (min^{-1})	0.34 ± 0.02	0.13 ± 0.01
K_M' (nM)	17.9 ± 5.6	16.7 ± 6.4
k_{cat}/K_M' ($min^{-1}·M^{-1}$)	1.89 x 10^7	0.81 x 10^7
K_{Mg} (mM)	2.2 ± 1.0	2.1 ± 0.8

Since we observed that the k_{cat} of δRzP1.2 is about 3 times less than that of δRzP1.1, while the K_M' is similar, we investigated whether an increased amount of Mg^{2+} in the cleavage reaction would affect the k_{cat} of δRzP1.2. Under single turnover conditions, in which the ribozyme and substrate concentrations were kept at 500 nM and 1 nM, respectively, we found that both ribozymes cleave their complementary substrates at Mg^{2+} concentrations as low as 1 mM, which is the estimated physiological concentration of Mg^{2+}. At this

concentration, the k_{obs} obtained were 0.11 ± 0.01 and 0.04 ± 0.01 min^{-1}, for δRzP1.1 and δRzP1.2, respectively (Fig. 3, panel B). A maximum k_{obs} for δRzP1.2 was observed when the concentration of Mg^{2+} was 10 mM. Higher concentrations of Mg^{2+} did not increase either the k_{obs} or the extent of cleavage for both ribozymes. We did not observe a decrease in the cleavage rate when higher concentrations of Mg^{2+} were used (*e.g.* 500 mM). The requirement for magnesium at half-maximal velocity (K_{Mg}) was 2 mM for both δRzP1.1 and δRzP1.2.

Substrate specificity

In order to compare the specificity of the *delta* ribozyme with various substrates, δRzP1.1 was used under single turnover conditions as described above. The cleavage reactions were performed with a trace amount of each substrate (< 1 nM) and 500 nM δRzP1.1. Under these conditions, the observed rates reflect the rates of cleavage without interference from either product dissociation or inhibition. For each substrate both the observed cleavage rate constants (k_{obs}) and the extent of cleavage were calculated and compared to those of the wild type substrate, as shown in Table 2.

Table 2

Cleavage activity of shorter or mismatched substrates as compared to the wild type substrate (SP1.1)

Substrates	Sequence	k_{obs}[a] (min^{-1})	Extent of cleavage[c] (%)	k_{rel}[d]	$\Delta\Delta G^{\ddagger}$[e] (kcal/mol)
Wild type substrate (S11-mer)	GGGC$G_5G_6G_7U_8C_9G_{10}G_{11}$	0.34 ± 0.02	48.3 ± 0.9	1	-
S10-mer	GGGCGGGUCG	0.022 ± 0.01	28.8 ± 4.3	0.063	-1.69
S9-mer	GGGCGGGUC	na[b]	na[b]	-	-
S8-mer	GGGCGGGU	na[b]	na[b]	-	-
SG5A	GGGCAGGUCGG	0.009 ± 0.002	20.0 ± 2.4	0.026	-2.25
SG5C	GGGCCGGUCGG	0.047 ± 0.017	1.7 ± 0.2	0.138	-1.22
SG6A	GGGCGAGUCGG	0.026 ± 0.006	5.8 ± 0.5	0.076	-1.59
SG6U	GGGCGUGUCGG	0.071 ± 0.026	3.7 ± 0.3	0.209	-0.96
SG7A	GGGCGGAUCGG	na[b]	na[b]	-	-
SG7U	GGGCGGUUCGG	na[b]	na[b]	-	-
SU8C	GGGCGGGCCGG	na[b]	na[b]	-	-
SU8G	GGGCGGGGCGG	na[b]	na[b]	-	-
SC9A	GGGCGGGUAGG	0.016 ± 0.007	8.2 ± 3.0	0.047	-1.88
SC9U	GGGCGGGUUGG	0.031 ± 0.005	21.2 ± 1.0	0.091	-1.48
SG10U	GGGCGGGUCUG	0.016 ± 0.002	8.4 ± 0.5	0.047	-1.88
SG11U	GGGCGGGUCGU	0.011 ± 0.001	32.1 ± 2.5	0.032	-2.12

[a] k_{obs} is the observed rate of cleavage calculated from at least two measurements. [b] na represents no detectable cleavage activity after a 3.5 hours incubation. [c] Cleavage extent (%) is obtained by fitting the data to the equation $A_t = A_\infty(1-e^{-kt})$, where A_t is the percentage of cleavage at time t, A_∞ is the maximum percentage of cleavage, and k is the rate constant. [d] k_{rel} is the relative rate constant as compared to that of wild type substrate. [e] $\Delta\Delta G^{\ddagger}$, the apparent free energy of transition-state stabilization, was calculated using the equation $\Delta\Delta G^{\ddagger} = RT\ln k_{rel}$, where T = 310.15 K (37 °C) and R = 1.987 $cal \cdot K^{-1}mol^{-1}$. Bold letters represent the nucleotides of wild type substrate recognized by δRzP1.1. The numbers in subscript indicate the nucleotides of wild type substrate which were individually altered to generate shorter or mismatched substrates.

Shorter substrates

Three shorter substrates containing 10, 9 and 8 nt were tested individually and compared to the 11-nt substrate (SP1.1) in which 7-nt base paired with δRzP1.1. The 10-, 9- and 8-nt substrates contain 6, 5 and 4 nt regions of complementary to δRzP1.1, respectively. We observed that the 10-nt substrate was cleaved with a k_{obs} of 0.02 ± 0.01 min^{-1} and a maximal cleavage of 28.8% (Table 2). We could not detect the cleavage product formed when the 9- and 8-nt substrates

were used, even after 3.5 hours incubation time.. The cleavage reactions were also carried out in the presence of 100 mM Mg^{2+}, instead of the 10 mM concentration used in a regular cleavage reaction. We observed no improvement in the values of the k_{obs} and the extent of cleavage for the 10-nt substrate, and still detected no cleavage for both the 9- and 8-nt substrates.

Mismatched substrates

We have generated a collection of substrates in which single mismatches were individually introduced into the P1 region of the substrate and then used in the cleavage reactions (Table 2). Mutation at position 5 resulted in at least a 9-fold decrease in k_{obs} as compared to that of SP1.1 (0.34 min^{-1}). However, for SG5A, in which A was substituted for G at position 5 of SP1.1, the extent of cleavage was only reduced by half. When this nucleotide was changed to cytosine, the cleavage was reduced almost to nil (*ca.* 1.7%). δRzP1.1 cleaved approximately 4% of the SG6A and SG6U substrates, in which A or U were substituted for G at position 6. The alteration of either position 7 or 8, located in the middle of the P1 stem, yielded uncleavable substrates (SG7A, SG7U, SU8C, SU8G). The k_{obs} was also drastically decreased when the C at position 9 was altered to A or U. The extent of cleavage was reduced to approximately 50%, when SC9U was used. The SG10U substrate, in which U was substituted for G at position 10, gave a similar result to SC9A. Finally, δRzP1.1 cleaved the substrate SG11U almost as well as SP1.1, although the k_{obs} was considerably slower (0.01 min^{-1}). The relative activity of each single mismatched substrate was calculated to obtain an apparent free energy of transition-state stabilization, $\Delta\Delta G^{\ddagger}$ (Chartrand, P. et al. (1997)

Biochemistry **36**, 3145-3150). We found that the values of $\Delta\Delta G^{\ddagger}$ range between -0.96 to -2.25 kcal·mol^{-1}. This apparent difference in activation energy was also observed when substrates of leadzyme were altered and used in a cleavage assay (Chartrand, P. et al. (1997) *Biochemistry* **36**, 3145-3150).

Equilibrium dissociation constant (K_d)

The four substrates containing a single mismatch either at position 7 or 8 which were not cleaved by δRzP1.1 were used to determine an equilibrium dissociation constant (K_d). Trace amounts of end-labeled substrates (SG7A, SG7U, SU8C or SU8G) were individually incubated with various concentrations of δRzP1.1 for the gel shift analysis as described above. To ensure that the dissociation equilibrium was reached, we incubated the reaction mixtures at various intervals. We found that the equilibrium was reached within 5 minutes, and that a longer incubation of 28 hours did not affect the measurement of K_d. Since SP1.1 can be cleaved under native gel electrophoresis conditions, we therefore used its analog which has a deoxyribose at position 4 (SdC4) to obtain the estimated K_d of the wild type substrate. This analog could not be cleaved by δRzP1.1 under the conditions used, and has been shown to be a competitive inhibitor of δRzP1.1 cleavage. An example of a gel shift analysis carried out for the analog is shown in Fig. 4. *Panel* ***A***. An autoradiogram of a nondenaturing polyacrylamide gel. The gel fractionation separated labeled substrate (free) from ribozyme-substrate complex (bound) at various concentrations of δRzP1.1. The positions of the top of the gel (ori) and xylene cyanol (XC) are indicated. *Panel* ***B***. The percentage of ribozyme-substrate complex (bound) were quantified and plotted

to fit a simple binding curve as described in Material and Methods with K_d = 31.9 ± 2.7 nM.

In this particular analysis, trace amounts of SdC4 (< 1 nM) were incubated with 11 concentrations of δRzP1.1 ranging from 5 to 600 nM. An autoradiogram of the resulting gel obtained by a Molecular Dynamic radioanalytic scanner is shown in Fig. 4, panel A. The bands of the bound SdC4 and the free SdC4 at each δRzP1.1 concentration were quantified, and the percentage of the bound SdC4 was plotted as shown in Fig. 4, panel B. The experimental data were fitted to a simple binding equation as described above to obtain K_d= 31.9 ± 2.7 nM. Similar experiments were performed for SG7A, SG7U, SU8C and SU8G. The substrates in which A or U were substituted for G at position 7 were observed to have a lower affinity for δRzP1.1 than those of the substrates in which the U at position 8 is altered. The higher K_d values obtained for SG7A (320 ± 20 nM) and SG7U (220 ± 60 nM), as compared to those of the analog (31.9 ± 2.7 nM), SU8C (36.1 ± 2.5 nM) and SU8G (71.5 ± 3.2 nM) are summarized in Table 3.

Table 3

Equilibrium dissociation constants of inactive single mismatched substrates as compared to the analog of the wild type substrate

Substrates	Sequence	K_d[a] (nM)	$\Delta G_{E.S}$[b] (kcal·mol^{-1})	K_{rel}[c]	$\Delta\Delta G_{E.S}$[d]
Analog substrate (SdC4)	GGGdC_4G$_5$G$_6$G$_7$U$_8$C$_9$G$_{10}$G$_{11}$	31.9 ± 2.7	-10.6	1	-
SG7A	GGGCGG**A**UCGG	320 ± 20	-9.2	10	-1.4
SG7U	GGGCGG**U**UCGG	220 ± 60	-9.4	7	-1.19
SU8C	GGGCGGG**C**CGG	36.1 ± 2.5	-10.6	1	0
SU8G	GGGCGGG**G**CGG	71.5 ± 3.2	-10.1	2	-0.43

[a] K_d is equilibrium dissociation constant obtained from fitting the data to the equation % bound substrate = [RZ]/K_d + [RZ], where [RZ] is the concentration of ribozyme, and K_d is the equilibrium dissociation constant. [b] $\Delta G_{E.S}$, the Gibbs-energy change, is calculated using the equation $\Delta G_{E.S} = -RT\ln K_d$, where T=310.15 K (37 °C) and R=1.987 cal K^{-1}·mol^{-1}. [c] K_{rel} > 1 indicates a destabilization effect of the given mismatched nucleotide. [d] $\Delta\Delta G_{E.S}$ is the relative change of Gibbs-energy obtained from either the equation $\Delta\Delta G_{E.S} = -RT\ln K_{rel}$, or from the difference between the values of $\Delta G_{E.S}$ of mismatched substrate and that of the analog. Bold letters and numbers in subscript represent the nucleotides and their positions in the analog recognized by ribozyme. The analog was designed to have a deoxyribonucleotide at positon 4 of the wild type substrate.

It was observed that the single mismatch introduced at position 7 disturbed the equilibrium of substrate-ribozyme complex formation to a greater extent than the mutation at position 8. The values of K_d were used in the determination of the free energy of substrate binding (Gibbs energy change, $\Delta G_{E.S}$). The mismatch at position 7 interfered with the stabilization of the substrate-ribozyme complex, resulting $\Delta\Delta G_{E.S}$ between -0.43 and -1.4 kcal·mol^{-1}.

Discussion

Delta ribozymes derived from the genome of HDV are of interest in the development of a gene regulation system in which the designed ribozymes would down-regulate the expression of a target gene. The facts that *delta* ribozymes are derived from HDV and that this pathogen naturally replicates in animal systems,

suggest that this catalytic RNA could be used to control gene expression in human cells. Like other ribozymes, the designed ribozyme should specifically cleave its target substrates while leaving other cellular RNA molecules intact. We designed a *trans*-acting *delta* ribozyme harboring a recognition sequence similar to the HDV antigenomic *delta* self-cleaving motif so as to have a minimal system for the study of the specificity of the base-pairing interaction between the *delta* ribozyme and its substrate.

The *trans*-acting *delta* ribozyme of the present invention, δRzP1.1, exhibited an activity with a cleavage rate of 0.34 min^{-1}, or a t_2 of 2 min, under pseudo-first order conditions. It was found that the extent of cleavage is approximately 60%, regardless of the concentration of ribozyme used, suggesting possibilities that i) a fraction of the substrate was bound to an inactive form of the *delta* ribozyme, ii) substrate was bound, instead to the P1 region of the ribozyme, to Cs of the 3′ of the ribozyme causing a misfold or a nonactive substrate/ribozyme complex, or iii) a portion of the ribozyme might adopt another conformation following substrate binding. Based on the latter hypothesis, the alternative form of ribozyme-substrate complex could undergo cleavage at a very low rate. We first investigated whether or not the presence of the alternative form could be a result of an infidelity of the T7 RNA polymerase transcription. Two batches of purified T7 RNA polymerase were tested using various amounts of enzyme and incubation times. We found that the transcripts produced by both batches of purified T7 RNA polymerase at the different incubating times exhibited a similar cleavage pattern and extent, suggesting that it is the nature of ribozyme transcripts to adopt an alternative form in the

reaction mixtures. A possible occurrence of misplaced or misfold substrate/substrate complex was dismissed since there is no evidence of other formed complex detected under non-denaturing gel electrophoresis. Finally, the possible occurrence of a slow cleaving form of *delta* ribozyme was assessed following cleavage reactions. We attempted to fit the experimental data using a multiphasic kinetic equation. Since we could not clearly describe the kinetics of our *trans*-acting *delta* ribozyme as biphasic or multiphasic reactions, we measured initial rates of cleavage for comparative purposes.

In order to summarize the *delta* reactions catalyzed by δRzP1.1 and δRzP1.2, free energy diagrams of the reaction coordinates were constructed (Fig. 5). The cleavage reactions for δRzP1.1 (P1, CCGACCU) and δRzP1.2 (P1, CCCAGCU) were carried out under similar conditions as described above. A standard state 1 M free substrate and ribozyme at 310.15 K (25°C) is assumed to be the same for both *delta* ribozymes. Gibbs energy changes of the enzyme-substrate complex formation ($\Delta G_{E.S}$) were calculated from an apparent K_M (K_M') using the equation, $\Delta G_{E.S} = -RT \ln K_M'$. The K_M' is 17.9 nM for δRzP1.1, and 16.7 nM for δRzP1.2; The $\Delta G_{E.S}$ is 10.9 kcal·mol^{-1} for δRzP1.1 and 11.0 kcal·mol^{-1} for δRzP1.2. Transition energy (ΔG^I) is calculated from k_{cat} using the equation, $\Delta G^I = -RT \ln k_{cat}.h/k_B.T$. The k_{cat} is 0.34 min^{-1} for δRzP1.1, and 0.13 min^{-1} for δRzP1.2; The ΔG^I is 18.8 kcal·mol^{-1} for δRzP1.1 and 19.4 kcal·mol^{-1} for δRzP1.2. *R*, Molar Gas Constant; *h*, Planck Constant; k_B, Boltzman Constant. Dashed lines indicate the values obtained for δRzP1.1.

The diagrams relate the two states in the cleavage reactions using kinetic parameters obtained under single turnover conditions. δRzP1.1 and δRzP1.2

differ in that they have two base pairs in the middle of the P1 stem interchanged. As expected, the free energies of substrate binding are virtually identical (-11 kcal·mol^{-1}). The base pair interchange in δRzP1.2 increased the value of $\Delta G^{\ddagger}$ by approximately 0.5 kcal·mol^{-1}. It is interesting to note that the free energy of the transition state was affected by the changes in the base pairing of the P1 stem. Since the kinetics of *delta* cleavage reactions appear to be affected by the particular combination of base pairs, it is very likely that, in addition to P1 base pairing, a tertiary interaction might also participate in substrate recognition. In this scenario the substrate-ribozyme complex would undergo a conformational transition, following formation of P1 stem, which involves tertiary interaction(s). These interactions might result in the positioning of the scissile bond in the catalytic center, a key step in the reaction pathway.

The substrate specificity of the *delta* ribozyme was studied using δRzP1.1. Firstly, we found that the *delta* ribozyme can cleave a substrate having a minimum of 6 nucleotides adjacent to the cleavage site. The k_{obs} of the 10-nt substrate is at least 10 times slower than that of SP1.1 (Table 2).

Secondly, to estimate the contribution of base-pairing interaction of the P1 stem to the cleavage reaction, a collection of a single-mismatched substrates were generated by introducing point mutations into the substrate sequence. Although there are several reports on the base pairing requirement of the P1 region (Perrotta, A.T., and Been, M. D. (1991) *Nature* **350**, 434-436; Been, M. D. (1994) *TIBS* **19**, 251-256), no extensive investigation has been performed on each individual nucleotide of either *cis*- or *trans*-

acting *delta* ribozymes. The determination of ribozyme specificity against various substrates was first attempted by comparing the apparent second-order rate constant (k_{cat}/K_M') of each substrate to that of wild type substrate. We found that the ribozyme cleaved single mismatched substrates very slowly, and gave a low percent cleavage (maximum of 2 to 20%) within the reaction time studied (3.5 h). As a consequence, the measurement of the apparent second order rate constants as a function of ribozyme concentration yielded values with a high margin of error. We thus reported the cleavage activity of the ribozyme against various single mismatched substrates in terms of extent of cleavage and k_{obs}, which at a high ribozyme concentration reflects the k_{cat} of the cleavage reaction. In all cases, we observed the decrease in cleavage extent which we suspected to be mainly due to the poor binding between the substrate and the ribozyme. The wobble base pair (G-U) at the cleavage site is required to maintain a high level of cleavage (Been, M. D. (1994) *TIBS* **19**, 251-256). Mismatches at this position which create either an A-U or a C-U pairing decreased the cleavage activity. It is interesting to note that the extent of cleavage decreases proportionally to the mismatches introduced into the 3' and 5' positions of the middle of the P1 stem.

The calculated free energy of transition-state stabilization ($\Delta\Delta G^{\ddagger}$) for each substrate, listed in Table 2, varies between -0.9 to -2.25 kcal·mol^{-1}. Each position of base pairing between the substrate and the ribozyme appears to affect the reaction pathway differently, at least with regard to transition state complex formation. If we assume that mismatched substrates yield the same level of $\Delta G_{E\cdot S}$, various end

points of cleavage for mismatched substrates could be resolved depending upon the height of the energy barrier level to be overcome in the transition state. To address these questions precisely, more experiments on the equilibrium binding constant and the internal equilibrium of the reactions are required. We have determined the calculated K_d of P1 duplex formation using the equation described by Serra and Turner (Serra, M. J. et al. (1995) *Methods in Enzymology* **259**, 242-261) to be 28.5 nM. By using an analog, we have shown that the K_d of the wild type substrate to its ribozyme is 31.9 nM. It is very interesting to note that the mismatch introduced at position U8 of the substrate has little effect on substrate binding affinity. However, the change completely eliminated cleavage activity. The mismatch introduced at position G7 of the substrate affected both the binding and chemical steps since it not only lowered the binding affinity of the substrate for the ribozyme, but also destroyed the cleavage activity. These findings suggest that some base pairs of the P1 stem have dual roles; participating in the substrate binding and subsequent steps leading a chemical cleavage. To address these findings more precisely some preliminary experiments have been carried out using the metal-ion induced cleavage method to study the tertiary structure of *delta* ribozyme. The data obtained to date suggests that positions U8 and G7 are likely involved in the formation of an essential metal-ion binding site. The mismatches introduced at either of the two positions destroyed the formation of this metal-ion binding site, a process which has been found to be highly associated with cleavage activity.

In accordance with the present invention, there is presented here evidence that aside from the base

pairing between the substrate and the ribozyme, tertiary interactions, especially ones involving the P1 stem, appear to dictate the reaction pathway of *delta* ribozyme. In order to fully comprehend how the cleavage reactions are governed, the elucidation of these tertiary interactions is essential.

Regarding recognition mechanism (e.g. substrate specificity), delta ribozyme as described with the present invention is unique among all ribozymes described thus far. Fig. 6 summarizes the substrate requirements for delta ribozyme to exhibit cleavage activity. Fig. 6A shows one of the example ribozyme-substrate complex characterized in the present work while Fig. 6B summarizes the substrate requirements for a RNA molecule composed of 11 long bases. The base pairing region includes 7 base pairs (position 5 to 11). A GU wobble base pair is required immediately.

3' to the cleavage site (position 5). The non-conventional base pair at position 8 required a pyrimidine (Y: U or C) in the substrate and a purine (R: A or G) in the ribozyme. This base pair is involved in a tertiary interaction with at least a third nucleotide belonging to the ribozyme. The nucleotide immediately 5' to the cleavage site should be a C, U or A (e.g. represented by the letter H). A preference is for a pyrimidine 5' adjacent to the cleavage site (position 4). Furthermore, the four nucleotides 5' to the cleavage site (e.g. position 1 to 4) must not base pair with the three opposite G of the ribozyme. Considering that residue U and C may base pair with G, a strong preference would be not to do two consecutive pyrimidine (Y: U or C) in position 1 to 4. As a consequence, we indicated the preference for purine (R: A or G) at position 1 to 3.

Thus, delta ribozyme is the only known catalytic RNA to involve both double- and single-stranded bases as well as an essential tertiary interaction for substrate specificity in order for cleavage activity to occur.

Figs. 7A and 7B Ribozymes composed by 1 or 2 (bimolecular) RNA strand cleaving a minimal model substrate. Fig. 7C Ribozyme cleaving a long substrate as a mRNA inherited to a disease or a viral RNA.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Variation of delta ribozyme

In addition to the sequence variations reported above, several derivatives of the delta ribozyme that exhibit efficient cleavage activity were designed. Fig. 7A illustrates an example of such a variation. Here, the ultrastable L4-loop (composition shown in right) was replaced by a relatively instable L4-loop. Surprisingly, this derivative was efficient as the construction with the ultrastable L4-loop.

EXAMPLE II

Bimolecular delta ribozyme

Fig. 7B illustrates another example of delta ribozyme derivative. In accordance with the present invention, a system was developed in which the ribozyme is separated in two fragments, referred to as a bimolecular system. More specifically, the L4-loop was removed and as a consequence the ribozyme is now formed by a RNA strand of 37 nucleotides (RZA) and a RNA strand of 20 nucleotides (RZB). Both RNA strands are deprived of catalytic activity. However, when RZA and RZB are folded together by formation of the P2 and P4

stems, the cleavage activity occurs. This is an original demonstration of such a bimolecular delta ribozyme. Because both RZA and RZB are relatively small, they can be chemically synthesized. Therefore, this bimolecular delta ribozyme give the advantage to allow introduction of any chemically modified nucleoside. As an example, each nucleotide of J4/2 junction domain was replaced by a deoxyribonucleotide (Fig. 7B). All RZB derivatives were actives, although at different levels depending on their efficiency to fold with RZA.

EXAMPLE III

Uses of the delta ribozyme as an anti-viral agent or a mRNA cutter

Fig. 7C illustrates a delta ribozyme that binds to a target RNA, such as a viral RNA, or an RNA crucial for the life cycle of a pathogen or an RNA inherited to a disease, based on the substrate specificity described in the present invention. Subsequently, the ribozyme will cleave the RNA target. In accordance with the present invention, delta ribozymes that cleave the mRNA coding for the HDV antigen were designed.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An enzymatic ribonucleic acid molecule consisting in a ribonucleotide sequence having an RNA substrate-cleaving enzymatic activity to cleave a separate RNA substrate at a cleavage site, which comprises:

a) an RNA substrate binding portion base pairing with the RNA substrate only 3' of the cleavage site in two consecutive RNA duplex having three Watson-Crick base pairs, wherein the two consecutive RNA duplex are separated by one pyrimidine forming a non-conventional Watson-Crick base pair with a purine of the ribozyme, and wherein said pyrimidine or said purine is involved in a tertiary interaction with a third nucleotide of the ribozyme;

b) an RNA substrate with a first nucleotide located 5' of the cleavage site is selected for the group consisting of U, A and C, and said first four nucleotides located 5' of the cleavage site remain single-stranded;

c) an enzymatic portion including part or all of the RNA substrate binding portion and having the enzymatic activity located 3' and/or 5' of the RNA substrate binding portion;

whereby base pairing of the enzymatic ribonucleic acid molecule with the separate RNA substrate causes cleavage of the RNA substrate at the cleavage site.

2. The enzymatic ribonucleic acid molecule of claim 1, wherein said ribonucleotide sequence is derived from hepatitis delta virus.

3. The enzymatic ribonucleic acid molecule of claim 1, wherein said RNA substrate include a sequence H↓GNNY'NN or H↓GNNY'NNN
wherein,

N is independently a nucleotide base forming Watson-Crick base pairs with the RNA substrate binding portion,

↓ is the cleavage site;

H is one base selected from the group consisting of A, U and C, wherein H is adjacent to the cleavage site and not base pairing to the ribozyme; and

Y' is a pyrimidine base forming a non-conventional Watson-Crick pair with the RNA ribozyme binding portion.

4. The enzymatic ribonucleic acid molecule of claim 3, wherein said RNA substrate sequence is selected from the group consisting of GGGC↓GNNUNNN, GGGC↓GNNCNNN, GGGU↓GNNUNNN, GGGU↓GNNCNNN, AAAC↓GNNUNNN.

5. The enzymatic ribonucleic acid molecule of claim 1, wherein said RNA ribozyme binding portion which base pairs with the RNA substrate 3' has the sequence NNR'NNU or NNNR'NNU,
wherein,

N is independently a nucleotide base forming Watson-Crick base pairs with the substrate, and

R' is a purine base forming a non-conventional Watson-Crick base pair with the substrate.

6. The enzymatic ribonucleic acid molecule of claim 5, wherein said RNA ribozyme binding portion may be selected from the group consisting of (5' to 3') NNNANNU and NNNGNNU.

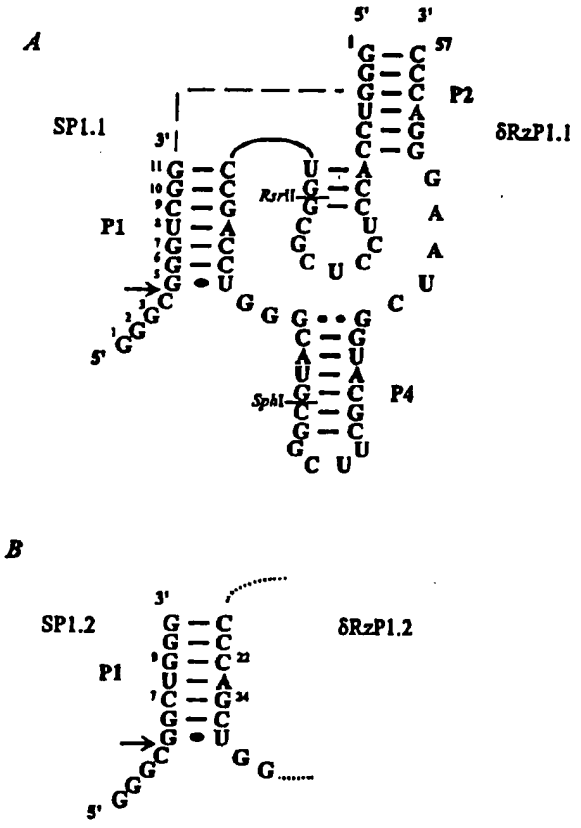
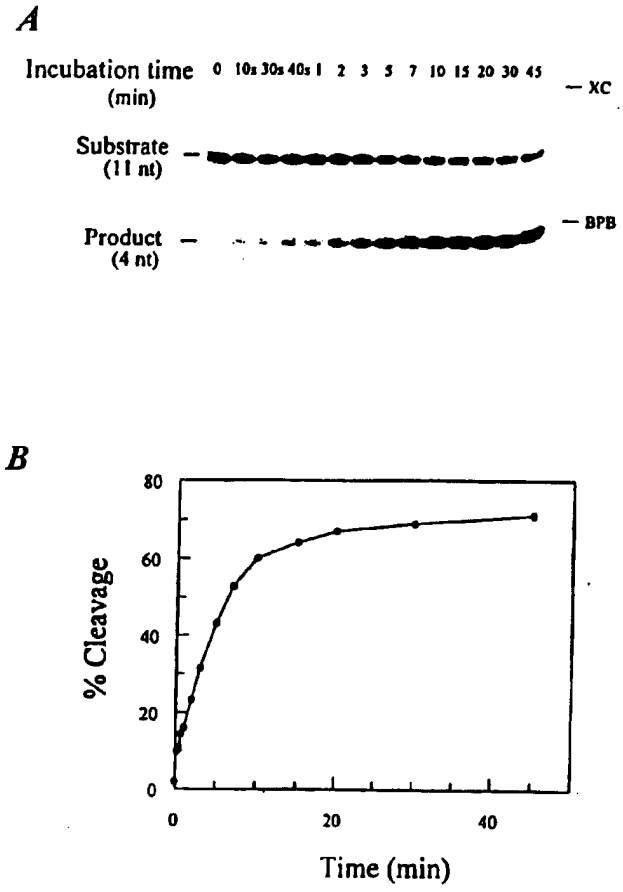


Fig. 1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Incubation time (min)
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XC
Substrate (11 nt)
Product (4 nt)
BPB
B
% Cleavage
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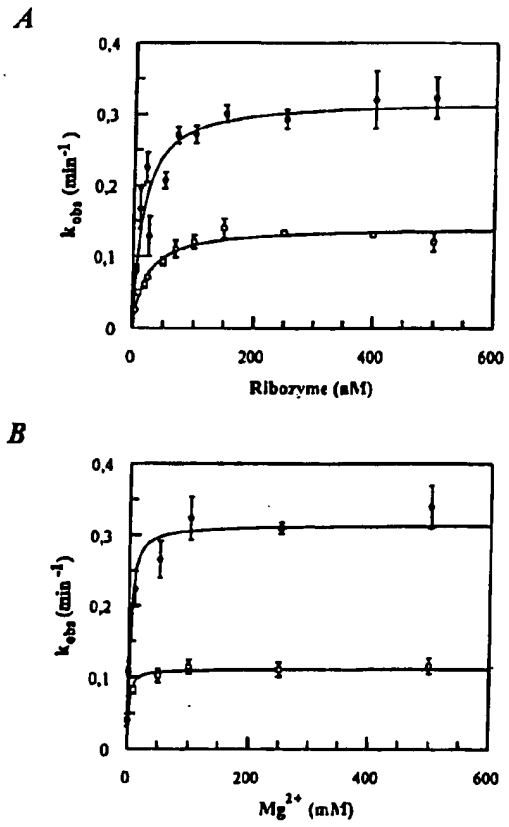
60
40
20
0
0
20
40
Time (min)

Fig. 2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0,1
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400
600
Ribozyme (nM)
B
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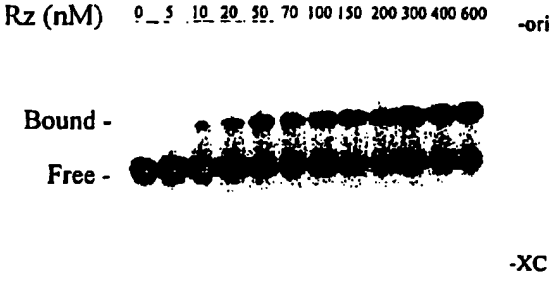
400
600
Mg^{2+} (mM)

Fig. 3

A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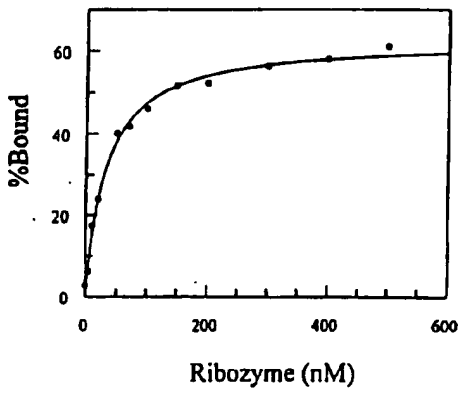
-ori
Bound -
Free -
-XC

B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40
20
0
0
200
400
600
Ribozyme (nM)

Fig. 4

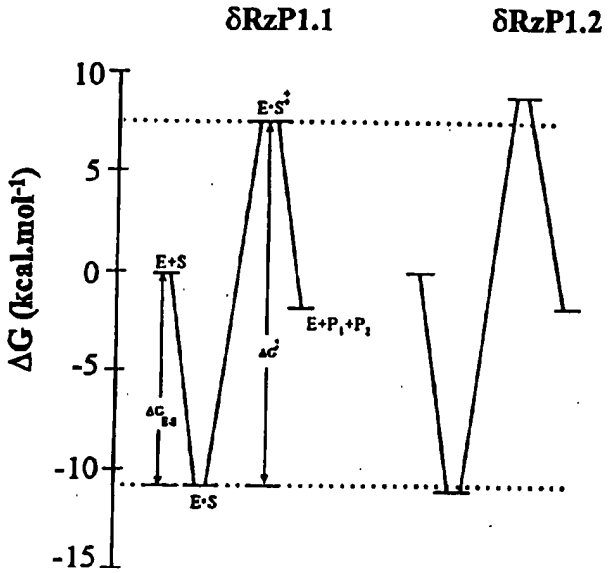


Fig. 5

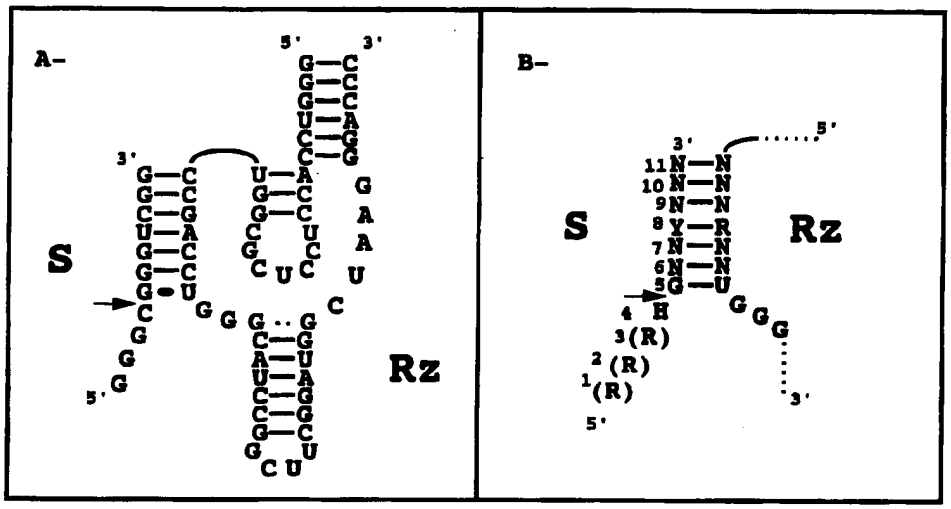
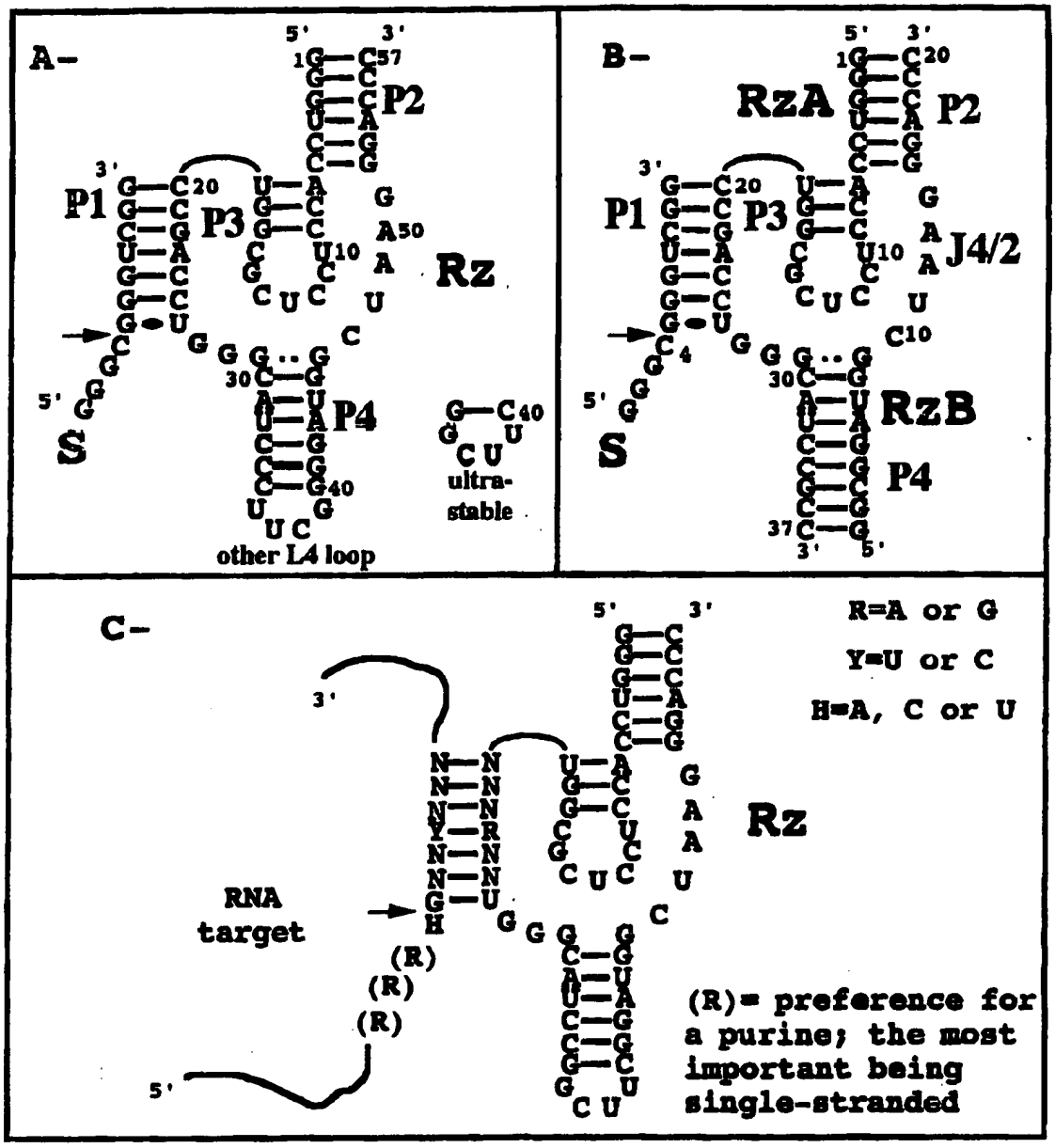


Fig. 6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P2
P3
P4
Rz
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other L4 loop
B-
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P2
P1
P3
J4/2
RzB
P4
S
C-
R=A or G
Y=U or C
H=A, C or U
Rz
RNA target
(R)
(R)
(R)
(R)= preference for a purine; the most important being single-stranded

Fig. 7